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Short Communication

Development of a novel internal positive control for Taqman® based assays

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Abstract

Development of rapid amplification assays for the detection and identification of biological threat agents has become a focus of diagnostic efforts in recent years. The use of real-time PCR assays as diagnostic tools depends upon two critical processes. First, nucleic acid purification must provide template that is both amplifiable and free of PCR inhibitors. Second, the assays themselves must be sensitive and specific for their nucleic acid targets. A differentiation must be made between results achieved due to the lack of target nucleic acid (true negatives) and those produced due to the inability to amplify target DNA (false negatives) so confidence in negative reactions is possible. False negatives can occur when inhibitors are present in the sample being tested, especially if clinical samples such as blood are analyzed. To address the problem of detecting inhibition in purified nucleic acids, an exogenous internal positive control (IPC) based on Taqman® chemistry was developed. A previously optimized assay was cloned and the primer and probe sites were mutated to produce novel sequences with no known homology to published sequence data. The IPC was sensitive to a variety of inhibitors, including hemoglobin, heparin, EDTA, humic acids, and fulvic acid. It was also equally sensitive to inhibition when labeled with either 6FAM or ROX dyes. In addition, the IPC was successfully multiplexed with agent specific assays without any loss in their sensitivity. The designed IPC assay has proven to be an effective tool for monitoring inhibitors of PCR and builds confidence in negative results obtained with agent specific assays.
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Keywords: PCR inhibitors; Exogenous internal positive control; Taqman® PCR; False negative

1. Introduction

PCR is an exquisitely sensitive methodology for the detection of nucleic acid. This high level of sensitivity creates an elevated risk of false positive results, those samples reported as positive when they are actually negative. Previous studies have focused on false positive reactions with regard to DNA contamination and amplicon carryover [1–3]. False negative results, samples reported as negative when they are actually positive, have also been examined [4–9] and could have severe consequences. For example, a negative test result for an infectious agent can influence therapeutic decisions, such as withholding antibiotic and antiviral drugs [8]. False negatives can be the result of failed PCR reactions due to expired reagents, poor technique, equipment failure, or the presence of nucleic acid amplification inhibitors such as EDTA, hemoglobin, heparin, fulvic acid, or humic acid [10–16]. The exact mechanism of inhibition is not clear for all of these compounds, but reports have clarified the means for some. EDTA chelates magnesium ions necessary for Taq polymerase activity [10]. Heparin binds to DNA [11,12], while hemoglobin releases iron ions, which are potentially related to its inhibitory effects [10]. In an attempt to detect inhibitors, several investigations report the incorporation of an internal control into PCR reactions [4–9,17]. These studies, however, utilize specific internal controls that incorporate some assay component, such as the same primer set.

In this study, a universal exogenous internal control was constructed for use with a variety of assays by employing site-directed mutagenesis of *Bacillus anthracis* Ames strain DNA. An existing assay targeting the protective antigen (PA) gene, located on the pX01 plasmid, was mutated to produce the novel internal positive control (IPC).

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This control was designed for dual use as either an independent assay or as a component of a multiplex diagnostic system. Evaluation was performed using a variety of PCR inhibitors including EDTA, hemoglobin, heparin, fulvic acid, soil humic acid, peat humic acid, and leonardite humic acid. Serial dilutions of each substance were tested to determine the concentration at which inhibition was relieved. In addition, IPC performance was evaluated with DNA extracted from whole blood spiked with gamma-irradiated *Yersinia pestis* to incorporate a sample set consisting of a complex matrix containing inhibitors simulating a real-world scenario.

2. Materials and methods

2.1. Bacillus anthracis PCR assay

The Taqman® based PCR assay for the *B. anthracis* PA gene mutated in this study was designed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). The amplification product of the assay is 153 base pairs (bp) in length. The product was mutated at the primer and the probe sites to create novel sequences with no known homology to published sequences.

2.2. Primer and probe design

The wild-type sequences of the PA primers and probe, BAPA3U, BAPA5L, and BAPA3P2A (Table 1), were randomized to produce new, unique sequences. The newly developed primers and probes were evaluated with the primer design software NetPrimer (PREMIER Biosoft International, Palo Alto, CA), to choose those with the best characteristics. A GenBank search was performed to ensure that ideal nucleotides for amplification did not have homology to published sequences (Table 3). The probe and primer sites of the *B. anthracis* PA assay were then mutated to produce the novel exogenous IPC. The probe-binding site was mutated first, followed by the sense and then the antisense primer binding sites.

2.3. Mutation of PA probe and primer sites

The mutation of each site was performed by using a five-step, site-directed PCR mutagenesis process [9,18] on wild-type amplification product (Fig. 1). The probe sequence was mutated first, thus creating a control specific for use with this PA assay using shared primers.

The first two steps of the process incorporated half of the mutated sequence into the generated PCR fragments. The first-half insertion of the probe site was performed using 1 ng of *B. anthracis* genomic DNA with primers 1 and 2 (BANPABIS1 and PA35PC1L) in one reaction and with primers 3 and 4 (PA35PC1U and BANPABIA1) in another reaction. Thermocycling conditions included 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The DNA fragments were then purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). The next two steps of PCR reactions allowed for full incorporation of the mutated sequence into the PCR products. In one reaction, the DNA fragment generated from primer set 1 and 2 was used as a template for amplification with primers 1 and 5 (BANPABIS1 and PA35PC2L). The other reaction used the DNA fragment generated from primer set 3 and 4 as a template for amplification with primers 4 and 6 (BANPABIA1 and PA35PC2U). Thermocycling conditions were identical to those described above. The DNA fragments were then purified with the QIAquick Gel Extraction Kit (Qiagen, Inc.). In the final round, both

Table 1
Sequence data for primers and probes

B. anthracis protective antigen diagnostic assay		
BAPA3U	5′ TTC AAG TTG TAC TGG ACC GAT TCT C 3′	
BAPA5L	5′ TCC ATC ATT GTC ACG GTC TGG 3′	
BAPA3P2A	5′ CGT AGG TCC AGC ACT TGT ACT TCG CTT 3′	
Mutagenesis primers		
PA35PC1U	5′ GCG ATC AAC TAC GTT CCA GAC CGT G 3′	Primer 3
PA35PC1L	5′ TCT CAG TCC ATC CGT TTT TCT TGA GTT C 3′	Primer 2
PA35PC2U	5′ ACG GAT GGA CTG AGA GCG ATC AAC TAC G 3′	Primer 6
PA35PC2L	5′ CGT AGT TGA TCG CTC TCA GTC CAT CCG T 3′	Primer 5
MOD31U	5′ CTG GAT TAT TAC CAA AAT AAA AAA G 3′	Primer 3
MOD31L	5′ TCG GTA ACA ACG ATC CAA TCC TTT T 3′	Primer 2
MOD32U	5′ CGT TGT TAC CGA CTG GAT TAT TAC C 3′	Primer 6
MOD32L	5′ GGT AAT AAT CCA GTC GGT AAC AAC G 3′	Primer 5
MOD51U	5′ TGG TAT GCG GAA TCC CTG ATT CAT T 3′	Primer 2
MOD51L	5′ GTT GTT GTC GAA CGT AGT TGA TCG C 3′	Primer 3
MOD52U	5′ CGA CAA CAA CTG GTA TGC GGA 3′	Primer 5
MOD52L	5′ TCC GCA TAC CAG TTG TTG TCG 3′	Primer 6
B. anthracis pX01 plasmid primers		
BANPABIS1	5′ CAA CGA GAA AAT CCT ACT GAA AAA G 3′	Primer 1
BANPA-BIA1	5′ GAA ATC ACT GTA CGG ATC AGA AGC 3′	Primer 4
Sequencing primers		
PCR II FOR	5′ CAG GAA ACA GCT ATG ACC 3′	Primer 1
PCR II REV	5′ TGT AAA ACG ACG GCC AGT 3′	Primer 4
Internal positive control assay		
IPC3L	5′ CGT TGT TAC CGA CTG GAT TAT TAC C 3′	
IPC5U	5′ TCC GCA TAC CAG TTG TTG TCG 3′	

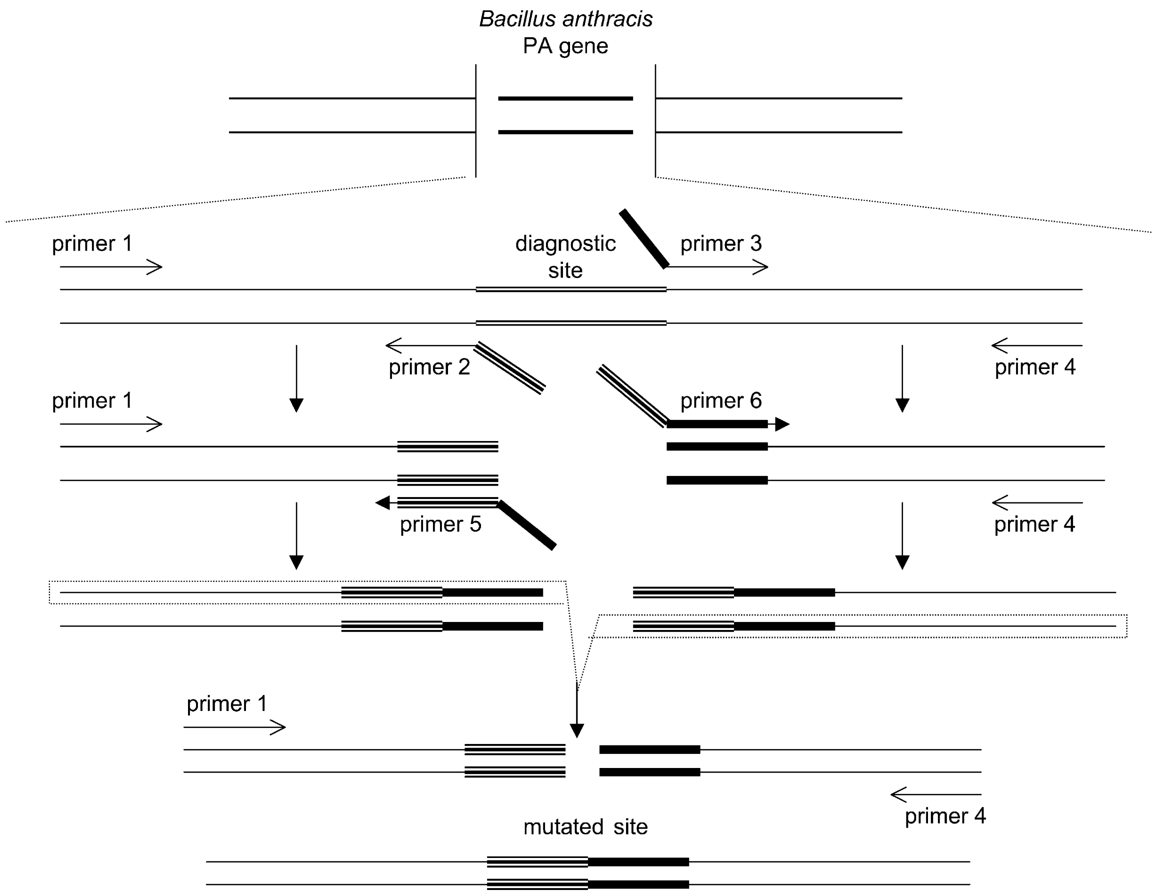

Fig. 1. Five-step site-directed PCR mutagenesis scheme.

amplicons produced from primers 1 and 5 and from primers 4 and 6 were used as primers for each other in an overlap extension reaction. Thermocycling conditions were identical to those described above, except only 20 cycles were performed. Immediately after extension, 1 μM each of primers 1 and 4 (BANPABIS1 and BANPABIA1) and 5U of additional AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA) were added to the reaction. Thermocycling conditions were as above for 10 more cycles producing a DNA amplicon fully incorporating the mutated sequence (Table 1, Fig. 1). The fragment containing the mutated probe site was cloned using the Original TA Cloning® Kit (Invitrogen Corp., Carlsbad, CA). The 347 bp amplification product was ligated into the pCR2.1 vector. Ligations were performed overnight at 14 °C according to manufacturer's instructions with 0.5 and 1.5 μl of amplified product. Transformations followed the manufacturer's specifications using the *Escherichia coli* INVαF′ One Shot Kit (Invitrogen Corp.). After ampicillin selection and blue/white screening, colonies were Taqman® PCR tested for the presence of the 347 bp insert. The screening primers were BAPA3U/5L with the probe IPCP35F. Once colonies containing the insert were identified, the plasmids were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) to ensure that the mutagenesis produced the sequence of interest. The selected plasmid was then purified with the Qiagen Plasmid Mini Purification Kit (Qiagen, Inc.) and sequencing was repeated for confirmation. The purified plasmid was used for the next round of mutations.

The BAPA3U primer sequence was mutated next. The PCR for the first-half insertion of the sense primer site used 1 ng of cloned DNA with the mutated probe site as template. Amplification was performed with primers 1 and 2 (PCR II FOR and MOD31L) in one reaction and with primers 3 and 4 (MOD31U and PCR II REV) in another reaction. Thermocycling conditions were identical to those described above. The DNA fragments were then purified with the QIAquick PCR Purification Kit (Qiagen, Inc.). The next two steps of PCR reactions allowed for full incorporation of the mutated sequence into the PCR products. In one reaction, the DNA fragment generated from primer set 1 and 2 was used as a template for amplification with primers 1 and 5 (PCR II FOR and MOD32L). The other reaction used the DNA fragment generated from primer set 3 and 4 as a template for amplification with primers 4 and 6 (PCR II REV and MOD32U). Thermocycling conditions were identical to those described above. The DNA fragments were then purified with the QIAquick Gel Extraction Kit

(Qiagen, Inc.). In the final round, both amplicons produced from primers 1 and 5 and from primers 4 and 6 were used as primers for each other in an overlap extension reaction. Thermocycling conditions were identical to those described above, except only 20 cycles were performed. Immediately after extension, 1 μM each of primers 1 and 4 (PCR II FOR and PCR II REV) and 5U of additional AmpliTaq® DNA polymerase (Applied Biosystems) were added to the reaction. Thermocycling conditions were as above for 10 more cycles producing a DNA amplicon fully incorporating the mutated sequence (Table 1, Fig. 1). The fragment containing the mutated probe and sense primer sites was cloned using the Original TA Cloning® Kit (Invitrogen Corp.). Following the BAPA3U primer site mutation, the screening primers were MOD32U (IPC3L) and BAPA5L. Once colonies containing the insert were identified, their plasmids were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) to ensure that the mutagenesis produced the sequence of interest. The selected plasmid was then purified with the Qiagen Plasmid Mini Purification Kit (Qiagen, Inc.) and sequencing was repeated for confirmation. The purified plasmid was used for the next round of mutations.

The BAPA5L primer sequence was mutated next. The PCR for the first-half insertion of the antisense primer used 1 ng of cloned DNA with the mutated probe and sense primer sites as template. Amplification was performed with primers 1 and 2 (PCR II FOR and MOD51U) in one reaction and with primers 3 and 4 (MOD51L and PCR II REV) in another reaction. Thermocycling conditions were identical to those described above. The DNA fragments were then purified with the QIAquick PCR Purification Kit (Qiagen, Inc.). The next two steps of PCR reactions allowed for full incorporation of the mutated sequence into the PCR products. In one reaction, the DNA fragment generated from primer set 1 and 2 was used as a template for amplification with primers 1 and 5 (PCR II FOR and MOD52U). The other reaction used the DNA fragment generated from primer set 3 and 4 as a template for amplification with primers 4 and 6 (PCR II REV and MOD52L). Thermocycling conditions were identical to those described above. The DNA fragments were then purified with the QIAquick Gel Extraction Kit (Qiagen, Inc.). In the final round, both amplicons produced from primers 1 and 5 and from primers 4 and 6 were used as primers for each other in an overlap extension reaction. Thermocycling conditions were identical to those described above, except only 20 cycles were performed. Immediately after extension, 1 μM each of primers 1 and 4 (PCR II FOR and PCR II REV) and 5U of additional AmpliTaq® DNA polymerase (Applied Biosystems) were added to the reaction. Thermocycling conditions were as above for 10 more cycles producing a DNA amplicon fully incorporating the mutated sequence (Table 1, Fig. 1). The fragment containing the mutated probe site was cloned using the Original TA Cloning® Kit (Invitrogen Corp.). After the BAPA5L primer site was mutated, the screening primers were MOD32U (IPC3L) and MOD52L (IPC5U). Once colonies containing the insert were identified, their plasmids were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) to ensure that the mutagenesis produced the sequence of interest. The selected plasmid was then purified with the Qiagen Plasmid Mini Purification Kit (Qiagen, Inc.) and sequencing was repeated for confirmation.

2.4. Restriction digest

One microgram of the purified plasmid DNA from the final clone was then linearized with EcoR I (10 Units/μl) (New England BioLabs Inc., Beverly, MA) in NEBuffer EcoR I at 37 °C for 2 h. The restriction enzyme was subsequently heat inactivated at 65 °C for 20 min.

2.5. Assay optimization

The IPC assay was optimized on both the Smart Cycler® (Cepheid, Sunnyvale, CA) and the R.A.P.I.D. (Idaho Technology, Inc., Idaho Falls, ID). All reactions were performed using 50 fg of IPC DNA, two step temperature cycling protocols, and probe concentrations standardized to specific background fluorescent values (200–300 fluorescent units on the Smart Cycler®, and 20–30 fluorescent units on the R.A.P.I.D. with a gain of 16). The temperature cycling profile consisted of a 2-minute 95 °C step to activate the Platinum Taq DNA polymerase (Invitrogen Corp.), followed by 45 cycles of 95 °C for 1 s and 65 °C for 20 s. First, $MgCl_2$ was tested at 3–7 mM per reaction, and then primer concentrations were evaluated from 100 nM to 1 μM in 100 nM increments. The optimum anneal/extend temperature was determined by testing the assay at 50, 55, 60, 65, and 70 °C. The final optimization step was a DNA titration with 100 fg, 10 fg, 1 fg, 500 ag, 250 ag, and 125 ag per reaction. On the Smart Cycler®, each reaction contained 1× PCR buffer (50 mM Tris pH 8.3; 25 μg/ml BSA, Idaho Technology, Inc.), 1× dNTP mix (0.2 mM dNTP, Idaho Technology, Inc.), primers, probe, 1 U of Platinum Taq DNA polymerase (Invitrogen Corp.), 1× Smart Cycler® Additive Reagent (1× SCAR 0.2 mM Tris pH 8.0; 0.2 mg/ml BSA; 150 mM Trehalose; 0.2% Tween 20, Cepheid), 5 μl of template DNA, and molecular biology grade water to bring total reaction volumes to 25 μl. Data acquisition and analysis were performed using Cepheid Smart Cycler® software Version 1.2d. Analysis settings consisted of a primary curve with a manual threshold of 10, background subtraction turned on, and boxcar average set to 5 cycles. On the R.A.P.I.D., each reaction contained 1× PCR buffer (50 mM Tris pH

8.3; 25 μg/ml BSA, Idaho Technology, Inc.), 1× dNTP mix (0.2 mM dNTP, Idaho Technology, Inc.), primers, probe, 1 U of Platinum Taq DNA polymerase (Invitrogen Corp.), 5 μl of template DNA, and molecular biology grade water to bring total reaction volumes to 20 μl. Sample curves were analyzed quantitatively using the LightCycler Data Analysis software (LCDA version 3.5.28) with 'Second Derivative Maximum' and baseline adjustment set to 'Arithmetic'. Qualitative analysis was performed using the R.A.P.I.D. Detector software version 2.0.7.

Table 2
Effect of various inhibitor concentrations using the FAM labeled IPC probe

Inhibitor	Concentration	End point fluorescence[a]		C_T[a]		Inhibition results[b]	
		Smart cycler	R.A.P.I.D.	Smart cycler	R.A.P.I.D	Smart cycler	R.A.P.I.D
EDTA	5 mM	0.761719078	−0.4687333	0.00	0.00	C	C
	2.5 mM	−0.496164697	1.7354	0.00	0.00	C	C
	1.25 mM	116.0457755	23.166533	36.57	35.59	R	R
	0.625 mM	163.132253	37.3512	36.00	34.08	R	R
	0.3125 mM	191.0560933	36.889	35.45	33.53	R	R
	H_2O	159.9769249	38.3496	35.93	33.27	+	+
Hemoglobin	0.6 μg/μl	4.643749391	−3.11	0.00	0.00	C	C
	0.3 μg/μl	3.396620723	−3.36	0.00	42.33	C	P
	0.15 μg/μl	−0.45828495	0.11	0.00	42.28	C	P
	0.075 μg/μl	ND	16.38	ND	38.20	ND	P
	0.06 μg/μl	36.81364124	ND	40.31	ND	P	ND
	0.0375 μg/μl	ND	111.59	ND	35.31	ND	R
	0.03 μg/μl	111.586515	ND	35.94	ND	R	ND
	0.01875 μg/μl	ND	125.66	ND	33.49	ND	R
	0.015 μg/μl	125.5696245	ND	35.63	ND	R	ND
	H_2O	125.7506154	42.01	35.58	33.33	+	+
Heparin	0.002 Units/μl	0.646091317	−1.2292	0.00	0.00	C	C
	0.001 Units/μl	−0.362003247	−1.2064	0.00	0.00	C	C
	0.0005 Units/μl	0.619221224	20.0488	0.00	>41.00	C	P
	0.00025 Units/μl	49.5407618	37.505867	38.75	35.27	P	R
	0.0002 Units/μl	73.12877993	ND	37.21	ND	R	ND
	0.000125 Units/μl	71.9054921	44.692267	36.95	34.18	R	R
	H_2O	102.1673165	46.840867	35.48	33.60	+	+
Soil humic acid	200 ppm	0.345537772	−0.1222	0.00	0.00	C	C
	100 ppm	0.715345742	−0.3761333	0.00	0.00	C	C
	50 ppm	1.105134458	−0.6655333	0.00	0.00	C	C
	25 ppm	31.90903407	−0.3078667	40.46	0.00	P	C
	20 ppm	ND	21.379133	ND	37.68	ND	P
	12.5 ppm	113.7710544	38.953067	35.69	33.87	R	R
	H_2O	123.6087935	46.090533	35.83	33.54	+	+
Peat humic acid	20 ppm	0.359540003	−0.5289333	0.00	0.00	C	C
	10 ppm	1.407237504	9.7892	0.00	41.54	C	P
	5 ppm	119.9774574	27.5671333	35.08	35.35	R	R
	2.5 ppm	79.19147932	41.9647333	35.77	33.41	R	R
	H_2O	113.8665515	43.4322	35.53	33.15	+	+
Leonardite humic acid	200 ppm	−0.182299768	−0.936	0.00	0.00	C	C
	100 ppm	15.11241402	1.0076667	42.81	43.67	P	P
	50 ppm	58.69080488	19.327733	38.68	38.45	P	P
	25 ppm	112.490771	40.081333	35.60	33.29	R	R
	20 ppm	109.5052036	32.836667	35.71	34.94	R	R
	H_2O	149.3741511	44.253933	35.42	33.86	+	+
Fulvic acid	50 ppm	ND	−0.459	ND	0.00	ND	C
	25 ppm	ND	9.464	ND	>40.00	ND	P
	20 ppm	0.15068026	ND	0.00	ND	C	ND
	12.5 ppm	ND	36.627	ND	34.37	ND	R
	10 ppm	61.94038776	ND	36.59	ND	R	ND
	6.25 ppm	ND	42.9956	ND	33.30	ND	R
	5 ppm	70.5988703	ND	36.13	ND	R	ND
	2.5 ppm	83.91532188	ND	35.70	ND	R	ND
	2 ppm	89.97937241	45.51587	36.02	33.48	R	R
	H_2O	86.35154594	47.95773	36.33	33.11	+	+

[a] End point fluorescence and C_T values: average of triplicates.
[b] Inhibition results: C, complete inhibition; P, partial Inhibition; R, complete relief of inhibition; ND, not determined; +, water blank for comparative value.

2.6. Inhibitor studies

The IPC assay was tested with seven different inhibitors: EDTA (Invitrogen Corp.), heparin (Sigma-Aldrich, St Louis, MO), hemoglobin (Sigma-Aldrich), fulvic acid, peat humic acid, leonardite humic acid, and soil humic acid (International Humic Substances Society, St Paul, MN). On the Smart Cycler®, 50 copies of the internal control clone were used, while 100 copies were used on the R.A.P.I.D. All inhibitors were initially tested with a 10-fold dilution series in triplicate. This was followed by a 2-fold dilution series in triplicate to determine the concentration of each compound at which inhibition was completely relieved (Table 2). Positive water blanks, performed in triplicate, were included in each run to provide a comparative value. All inhibitor experiments were performed with the IPC probe labeled with both FAM and ROX dyes (Biosearch Technologies, Inc., Novato, CA) (Data not shown).

2.7. Multiplexing experiments

Ten-fold serial dilutions (10 pg–10 ag) of quantified DNA were prepared and run in triplicate to determine assay sensitivity. These assays were optimized for primer, probe, and $MgCl_2$ concentrations, as well as annealing temperature. The same limit of detection experiments were repeated while multiplexing with the IPC assay. All of these experiments were performed using the Cepheid Smart Cycler®. In the diagnostic assays, FAM labeled probes were used, while for the IPC assay, ROX labeled probes were used.

2.8. Gamma irradiated Yersinia pestis spiked blood

Whole blood was collected in EDTA, sodium citrate or heparin tubes and subsequently spiked with ten-fold dilutions of gamma irradiated *Y. pestis* at a starting concentration of 10^5 cfu/ml. Ten microliter aliquots of each dilution were spotted in triplicate onto four triangular tips of an IsoCode Stix device (Schleicher and Schuell, Keene, NH) and dried for 15 min using a general-purpose heat gun. Triangles were detached into sterile microcentrifuge tubes and 1 ml of molecular biology grade water was added to each tube. Triangles were washed by pulse vortexing three times, for a total of 5 s, and the wash water was removed. One hundred microliters of sterile molecular biology grade water was then added, ensuring complete submersion of the DNA containing triangles. The nucleic acid was eluted by heating at 95 °C for 15 min in an Eppendorf Thermomixer with an agitation setting of 6. After a brief centrifugation, eluates were transferred to sterile microcentrifuge tubes for analysis by real-time PCR using the R.A.P.I.D. (Idaho Technology, Inc.). Undiluted and dilutions of the eluates were analyzed for the presence of inhibitors using the IPC assay. Both sets of eluates were also tested with a pPCP1 *Y. pestis* specific assay.

3. Results

A prerequisite for an effective universal IPC is a lack of homology to other known sequences. While there is some minor homology with both primers (Table 3), the IPC probe sequence appears to be unique. Furthermore, loss of performance or non-specific amplification in the presence of human DNA has not been observed. Optimal assay conditions consisted of 3 mM $MgCl_2$, 0.5 μM of each primer, and an anneal/extend temperature of 65 °C. The final concentration of IPC DNA was titrated to the lowest concentration that yielded consistent C_T and endpoint fluorescence values. The final concentration of IPC DNA used was instrument specific, 250 ag of DNA per reaction on the Smart Cycler® (50 gene copies) and 500 ag per reaction on the R.A.P.I.D. (100 gene copies).

The IPC assay was then tested with seven different inhibitors. All inhibitors were initially tested with a 10-fold dilution series followed by a 2-fold dilution set to determine the concentration of each compound at which inhibition was completely relieved and to confirm the sensitivity of the assay to inhibition (Table 2). On both platforms, the level where inhibition was completely relieved was comparable for all tested substances. Similar results were seen with both FAM and ROX labeled probes, indicating that various dyes could be used for the assay (Data not shown).

The IPC was multiplexed with other diagnostic assays to assess the ability for simultaneous detection of a biological agent and monitoring of PCR inhibition. Multiplex reactions were performed with conditions found to be optimal for each assay, without any further optimization in the presence of the IPC. Due to the larger amplicon size of the IPC assay (compared to the agent specific assays) and the limiting concentration of IPC DNA, there was very little effect on

Table 3
IPC primers and probe sequence homology

Assay oligo	Sequence	Accession #	E-value	In GenBank
IPC5U	Zebrafish DNA sequence from clone CH211-103D22 in linkage group 10	AL732562	1.4	20-Jun-03
IPC5U	Mouse DNA sequence from clone RP23-52D18 on chromosome 2	AL928877	5.7	29-Jan-03
IPC5U	*Physarum polycephalum* rRNA small subunit (SSU rRNA) gene	X13160	5.7	06-Jul-89
IPC3L	*Homo sapiens* chromosome 7 clone RP11-348A15, complete sequence	AC009364	0.85	15-Mar-03
IPC3L	Human chromosome 7 clone RP11-297N5, complete sequence	AC018643	0.85	17-Nov-00

Table 4
Limit of detection of agent specific assays with and without IPC

Organism	Gene target	LOD	
		w/ IPC	w/o IPC
Bacillus anthracis	Protective antigen	10 fg	10 fg
Bacillus anthracis	Capsule B	1pg	100 fg
Bacillus anthracis	Capsule B	100 fg	10 fg
Bacillus anthracis	Variable region with repetitive sequence	10 fg	10 fg
Brucella	Outer membrane protein 25	1 fg	1 fg
Clostridium botulinum	Botulinum enterotoxin A	100 fg	100 fg
Francisella tularensis	Tularensis lipoprotein	10 fg	1 fg
Francisella tularensis	Outer membrane protein A	10 fg	10 fg
Orthopox sp.	Hemagglutinin	100 fg	100 fg
Variola	Variola hemagglutinin	100 ag	100 ag
Yersinia pestis	Plasminogen activator	1 fg	1 fg
Yersinia pestis	Pesticin immunity assay	10 fg	10 fg

the limit of detection of the agent specific assays (Table 4). There were only three assays that had a significant loss in detection limit. Two of these were *B. anthracis* Capsule B assays, and the other targeted the *Francisella tularensis* lipoprotein gene. The limits of detection for the other nine assays tested were not affected, indicating that multiplexing was feasible.

An important requirement for success of an inhibition assay is to be more responsive to inhibitors than the assays being used for agent identification. To address this issue, DNA was extracted from whole blood spiked with a known concentration of gamma-irradiated *Y. pestis* cells. The samples were then tested with both the IPC assay and pPCP1 *Y. pestis* specific assay to compare performance (Table 5). Previous work demonstrated that *Y. pestis* DNA could be detected consistently from samples containing at least 40 cfus when tested using the R.A.P.I.D. platform (Data not shown). Results indicated that the IPC assay was as sensitive to inhibition as the *Y. pestis* specific assay when DNA was purified from whole blood. The IPC assay indicated the occurrence of inhibition in all cases where negative results were observed for the specific assay at or above its limit of detection. If inhibition had not been monitored in this circumstance, these would be false negative results. At high concentrations of bacterial cells, inhibition was observed for several samples; however, the *Y. pestis* specific assay produced positive results because the concentration of template overcame the inhibitory effects of the matrix. A 1:2 dilution relieved inhibition in all but one sample resulting in positive *Y. pestis* detection at the concentrations tested. The one exception required further dilution (1:4) to completely relieve inhibition. These results indicate that the IPC assay is functioning to assess inhibition and correlates well with the inhibitor effects on the target assay.

Table 5
Inhibition and pPCP1 specific assay results of DNA extracted from whole blood collected in EDTA, heparin, or sodium citrate tubes spiked with gamma-irradiated *Y. pestis*

cfu/ml	cfu/ extraction	EDTA						Heparin				Sodium citrate			
		Undiluted		1:2 Dilution		1:4 Dilution		Undiluted		1:2 Dilution		Undiluted		1:2 Dilution	
		Inhibition	pPCP1	Inhibition	pPCP1	Inhibition	pPCP1	Inhibition	pPCP1	Inhibition	pPCP1	Inhibition	PPCP1	Inhibition	pPCP1
1×10^5	4×10^3	Y	−	N	+	ND	ND	Y	+	N	+	Y	+	N	+
		Y	−	N	+	ND	ND	N	+	N	+	Y	+	N	+
		Y	−	N	+	ND	ND	Y	−	N	+	Y	−	N	+
1×10^4	4×10^2	Y	−	N	+	ND	ND	Y	+	N	+	Y	+	N	+
		Y	+	N	+	ND	ND	Y	+	N	+	Y	−	N	+
		Y	−	Y	+	N	+	Y	−	N	+	Y	−	N	+
1×10^3	4×10^1	Y	−	N	+	ND	ND	Y	+	N	+	Y	+	N	+
		Y	−	N	+	ND	ND	N	+	N	+	Y	−	N	+
		Y	−	N	+	ND	ND	Y	−	N	+	Y	−	N	+
1×10^2	4×10^0	Y	−	N	−	ND	ND	N	+	N	−	Y	−	N	−
		N	−	N	−	ND	ND	Y	+	N	−	Y	+	N	−
		Y	−	N	−	ND	ND	Y	−	N	+	Y	−	N	+
0	0	Y	−	N	−	ND	ND	Y	−	N	−	Y	−	N	−
		Y	−	N	−	ND	ND	Y	−	N	−	Y	−	N	−
		Y	−	N	−	ND	ND	Y	−	N	−	Y	−	N	−

ND, not determined; +, Positive; −, Negative; Y, Inhibition; N, No Inhibition.

4. Discussion and conclusions

In designing the internal control, several parameters were taken into consideration including standard primer design characteristics such as T_m, base composition, elimination of secondary structures, duplexes, and primer-dimer formation. The probe was also designed to omit a G on the 5′ end, a requirement of fluorescent probe design. In addition, the amplicon size was a major consideration. Typically, smaller amplification products are kinetically favored and amplify more efficiently than larger products. Therefore, we designed the IPC assay amplicon to be equivalent in size to our largest agent specific assay, 153 bp. The rationale behind this choice was that target assays with smaller amplification product sizes would amplify more efficiently than the internal control. This was important for two reasons: (1) the agent specific assay needs to preferentially amplify in multiplex reactions and (2) the IPC assay has to be at least as sensitive to inhibition as the agent specific assay. Once the new primer and probe sequences were designed to meet the criteria outlined, a GenBank search was performed to ensure that non-specific hybridization would not occur.

The optimization of the IPC assay ensured a fairly robust signal with consistent results while maintaining sensitivity to inhibition. Taqman® PCR works best at elevated $MgCl_2$ levels as it enhances Taq enzyme exonuclease activity and all of the other USAMRIID Taqman® assays use more than 3 mM $MgCl_2$. The low level chosen, 3 mM, works best for detecting inhibition by ion chelators such as EDTA. Furthermore, the optimization was performed to be used as a stand-alone assay; however, the universal internal control was designed so it could be incorporated for multiplexing with any other Taqman® assay.

Testing for inhibition with the IPC assay resulted in three outcomes: (1) complete inhibition, producing a flat line which indicated the assay did not work at all; (2) partial inhibition, where the cycle threshold (C_T) is delayed and/or endpoint fluorescence (EPF) is reduced; or (3) inhibition relief/no inhibition, when the C_T and EPF are comparable to the H_2O blank. The criterion to determine inhibition was based on a C_T shift or reduction in EPF. C_T is a measure of sample input, while EPF is an indicator of the robustness or efficiency of the amplification reaction. A C_T shift of 3 cycles is quantitatively equivalent to approximately a one-log loss in copy number (detection limit). Therefore, a C_T shift of ≥ 3 cycles beyond the water blank was considered inhibitory. A drop in EPF signifies a decline in amplification efficiency and is observed with PCR inhibition. Experience with the IPC during development demonstrated that a 50% drop in EPF correlated well with inhibitory samples but was greater than the normal EPF fluctuations observed among samples. Therefore, inhibition was indicated when a sample had a C_T shift of ≥ 3 cycles or a reduction in EPF of $\geq 50\%$ when compared to water controls. If inhibition is observed in a sample, two-fold dilutions are performed until inhibition is relieved. C_T shifts of <3 cycles or $<50\%$ loss of EPF were not considered significant, and dilution of these samples would unnecessarily dilute out the target DNA. All inhibitors tested in this study had a 100% correlation between a 3 C_T shift and a 50% drop in EPF (Table 2).

The inhibition assay was assessed for ‘real-world’ utility with samples of whole blood (EDTA, heparin, and sodium citrate tubes) spiked with limiting amounts of *Y. pestis*. Previous work established 1×10^3 cfu/ml (4×10^1 cfu/extraction) as the detection limit of the *Y. pestis* assay. Blood is a particularly complex matrix with a number of known PCR inhibitors, including hemoglobin and lactoferrin [10]. Likewise, components of the collection tubes, such as EDTA and heparin, are also inhibitory to PCR. After extraction of the blood with an Isostix procedure, a direct correlation could be observed between the presence of inhibition and the ability to detect *Y. pestis* in the samples. At concentrations of *Y. pestis* within the detection limit of the assay, all samples negative for *Y. pestis* were also found to be inhibitory by the IPC. All inhibition could be relieved by 2-fold serial dilution of the samples, and *Y. pestis* could be detected at the dilution where inhibition was relieved. At higher concentrations of agent, inhibition could be overcome in some instances. The critical finding was that false negative results (sample is negative, inhibition is not observed) were not observed in this study across all three blood collection tubes. The blood work demonstrates the utility of the IPC assay to prevent false negatives by identifying inhibition as the causative factor in a negative result.

In this study a novel internal control was designed, developed, and tested to be used in Taqman® based PCR assays. It is beneficial because it can be used as either a stand-alone control or one that can be multiplexed with other target assays. The method is simple, straightforward, and allows for confirmation of negative results as true negatives.

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